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PATENT APPLICATION

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TITLE: STABLE, AEROSOLIZABLE SUSPENSIONS OF PROTEINS IN ETHANOL

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STABLE, AEROSOLIZABLE SUSPENSIONS OF PROTEINS IN ETHANOL

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Application No. 60/250,491 [Attorney Docket No. 22112(1)P], filed December 1, 2000.

BACKGROUND OF INVENTION

This invention relates generally to aerosolizable stable suspensions of proteins in ethanol. The suspensions of the invention are particularly suited for inhalation delivery using electrostatic or electrohydrodynamic aerosol devices to produce the aerosol.

Pulmonary delivery of therapeutic agents by means of inhaled aerosols is an area of increasing importance in the biotechnology and pharmaceutical industries. Electrostatic or electrohydrodynamic devices which are capable of generating inhalable aerosols with certain preferred properties frequently require liquid formulations containing one or more aqueous solvents such as water, an organic solvent such as an alcohol, or a mixture of an aqueous and an organic solvent.

Biological molecules such as proteins are frequently difficult to formulate in certain solvents or solvent systems (e.g., mixtures of water and organic solvents) because organic solvents tend to compromise the stability of the protein in solution. Proteins dissolved or suspended in liquid solvent systems typically suffer from chemical or physical degradation, thereby resulting in a formulation with little or no shelf-life. Stabilization of the protein in a given solvent system is therefore necessary to sustain the activity of a given protein.

In order to be useful in a pharmaceutical application, proteins require processing and storage conditions, which do not diminish a given protein's biological function. To prevent the loss of native conformation, proteins must be protected from the chemical decomposition (e.g. by deamidation) and physical instability, which results from the disruption of non-covalent

interactions. Aggregation, precipitation, and adsorption (especially on hydrophobic surfaces) are examples of such disruption.

One of the most important and commonly used therapeutic proteins is insulin. Insulin tends to polymerize and form aggregates, which prevent the delivery of insulin in certain drug delivery systems. This aggregated insulin may not have required pharmacological properties and may induce abnormal immune response (*Chawla et al., Diabetes 34: 420-425, 1985*). Thus, maintenance of insulin's biological activity is essential for more traditional insulin administration such as portable/implantable continuous infusion pumps and controlled release polymeric devices and systems. Furthermore, insulin aggregation leads to significant reductions in biological potency and obstruction of delivery routes, thereby creating serious complications for drug delivery systems and diminishing the patient's abilities to control their blood glucose levels." *Sluzky et al., Biotechnology and Bioengineering 40: 895-903 (1992).* Thus, there is a need for a method for stabilizing insulin and other proteins in liquid formulations utilized in electrostatic or electrohydrodynamic aerosol drug delivery systems.

Stabilization methodologies for certain biological molecules are known in the art. For example, WO 98/29097 discloses compositions for increasing bioavailability through muscosal delivery, which includes mixtures of bioactive agents, and hydrophobically derivatized carbohydrates in powdered form. Similarly, WO 99/33853 discloses derivatized carbohydrates, which can be used to form a variety of materials, including solid delivery systems.

U.S. Patent 4,439,421 issued to Hooper et al. (1984) discloses a stabilized gamma globulin concentrate in dry form, which utilizes polysaccharides including, branched and unbranched polymers of five and/or six carbon sugars. U.S. Patent 5,547,873 issued to Magneson et al. (1996) discloses a composition for stabilizing proteins for long-term dry storage, which includes a glass-forming sugar.

While being effective at stabilizing certain biomolecules under certain conditions, the related art deals primarily with dry power or other solid systems, and do not offer methods or compositions for stabilizing biomolecules in liquid solvent systems suitable for use in electrostatic or electrohydrodynamic aerosol devices and systems.

These and other deficiencies of the prior art are overcome by the present invention, which provides methods and compositions for stabilizing proteins in liquid ethanol formulations that may be aerosolized aerosol drug delivery devices. These novel formulations are stable over extended periods, and as such, provide distinct advantages over other methods of formulating proteins in liquids that are compatible with aerosol drug delivery devices.

SUMMARY OF INVENTION

The present invention is directed to a stable suspension of a biologically active protein suited for aerosol delivery to the respiratory tract of a patient in need of treatment comprising particles of said protein suspended in ethanol. In a preferred embodiment, the invention describes a stable suspension of insulin useful for aerosol delivery to the lungs of a patient in need of treatment comprising particles of a pharmaceutically effective amount of insulin suspended in ethanol. The stable ethanol suspensions of the invention may optionally contain up to about 20% (V/V) of a pharmaceutically acceptable formulation additive such as glycerol, propylene glycol and polyethylene glycol as well as minor amounts (from about 0.05% to about 5.0% W/V) of a pharmaceutically acceptable excipient.

The invention is further directed to a method of delivering a therapeutically effective amount of a protein to the respiratory tract of a patient in need of treatment which comprises producing an aerosol of a stable liquid suspension of said protein using an electrohydrodynamic spraying means wherein said liquid suspension comprises particles of said protein suspended in ethanol.

According to the present invention, one method for stabilizing a protein in ethanol includes the step of making a suspension of a protein in ethanol by means of over-saturating ethanol with the protein, thereby promoting the formation of crystals of the protein. In an alternative method, protein crystals are added to ethanol at a high concentration to form a suspension, whereby the protein remains crystallized and does not dissolve.

DETAILED DESCRIPTION OF INVENTION

The present invention is directed to a stable suspension of a biologically active protein suited for aerosol delivery to the respiratory tract of a patient in need of treatment comprising particles of said protein suspended in ethanol. In a preferred embodiment, the invention describes a stable suspension of insulin useful for aerosol delivery to the lungs of a patient in need of treatment comprising particles of a pharmaceutically effective amount of insulin suspended in ethanol. The stable ethanol suspensions of the invention may optionally contain up to about 20% (V/V) of a pharmaceutically acceptable formulation additive such as glycerol, propylene glycol and polyethylene glycol as well as minor amounts (from about 0.05% to about 5.0% W/V) of a pharmaceutically acceptable excipient.

The invention is further directed to a method of delivering a therapeutically effective amount of a protein to the respiratory tract of a patient in need of treatment which comprises producing an aerosol of a stable liquid suspension of said protein using an electrohydrodynamic spraying means wherein said liquid suspension comprises particles of said protein suspended in ethanol.

According to the present invention, one method for stabilizing proteins in an ethanol entails making a suspension of a protein such as insulin in ethanol by means of over-saturating the ethanol with the protein. Preferably, the protein molecules are dissolved or suspended in ethanol, at concentrations, which promote the formation of crystals of the protein. In an alternative method, protein crystals are added to an ethanol at a high concentration to form a suspension, whereby the protein remains crystallized and does not dissolve. In a preferred embodiment of the present invention, the biological molecule is insulin.

As used herein, the term "stable" or "stability" is used to mean preservation of the biological activity of a protein.

The term "suspension" as used herein is given its ordinary meaning and refers to particles of protein or aggregates of particles of protein suspended in a liquid.

The stable suspensions of the invention are useful for preparing aerosols for the delivery of therapeutic proteins to the respiratory tract. The

term "respiratory tract" includes the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conductive airways. The terminal bronchioli then divide into respiratory bronchioli, which then lead to the ultimate respiratory zone, the alveoli, or deep lung. Gonda, I. "Aerosols for delivery of therapeutic and diagnostic agents to the respiratory tract," in Critical Reviews in Therapeutic Drug Carrier Systems, 6: 273-313, (1990). Usually, the deep lung, or alveoli, is the primary target of inhaled therapeutic aerosols for systemic delivery.

The term "biologically active protein" includes proteins and polypeptides that are used for diagnostic and reagent purposes as well as proteins and polypeptides that are administered to patients as the active drug substance for treatment of a disease or condition. Contemplated for use in the compositions of the invention are proteins and polypeptides such as enzymes, e.g., ascorbate oxidase, peroxidase, catalase, glucose oxidase, chymotripsin, lactate dehydrogenase and glucose-6-phosphate dehydrogenase; antibodies, e.g. Herceptin® (trastuzumab), Orthoclone OKT®3 (muromonab - CD3); hormones, e.g., insulin and human growth hormone (HGH); growth factors, e.g., fibroblast growth factor (FGF), nerve growth factor (NGF), human growth hormone releasing factor (HGHRF), and cytokines, e.g., leukemia inhibitory factor (LIF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-9 (IL-9), oncostatin-M (OSM), and Factor VIII.

In addition to enzymes and antibodies used in diagnostic tests or in *in vitro* assays, the term "biologically active" includes proteins that are administered to a patient in a "pharmaceutically effective amount" to treat a disease or condition. As would be recognized by one skilled in the art, by "pharmaceutically effective amount" is meant an amount of a pharmaceutical protein having a therapeutically relevant effect on the disease or condition to be treated. A therapeutically relevant effect relieves to some extent one or more symptoms of the disease or condition in a patient or returns to normal either partially or completely one or more physiological or biochemical

parameters associated with or causative of the disease or condition. Specific details of the dosage of a particular active protein drug may be found in its labeling, i.e., the package insert (see 21 CFR § 201.56 & 201.57) approved by the United States Food and Drug Administration.

The stable protein suspensions of the invention may optionally contain up to about 20.0% (V/V) of a formulation additive and preferably no more than about 10.0 % (W/V). The term "formulation additive" refers to a pharmaceutically acceptable organic liquid, which may be added to the ethanol suspensions of the invention to enhance the sprayability of the liquid protein suspension using an electrostatic spraying means. Contemplated for use, as formulation additives in the stable protein suspensions of the invention are polyhydric alcohols, e.g., glycerol and propylene glycol and polyglycols such as polyethylene glycol. The formulation additive should be pharmaceutically acceptable, that is, recognized by the US Food and Drug Administration as being safe for use in humans.

As would be recognized by the skilled artisan, the stable protein suspensions of the invention may optionally include "minor amounts", that is from about 0.05% to about 5.0% W/V and preferably from about 0.05% to from about 1.0% of a pharmaceutically acceptable excipient. Pharmaceutically acceptable excipients are those recognized by the FDA as being safe for use in humans. Additives such as, surfactants, e.g., ethoxylated dodecyl alcohol, antioxidants, e.g., Vitamin E and ascorbic acid, antimicrobials, e.g., parabens and suspending agents, e.g., povidone are contemplated for use herein.

While the selection of any particular excipient is within the skill of the art, as will be recognized the decision regarding whether to add an excipient and if so which one, will be made taking into account the purpose of the excipient in a specific ethanol protein suspension.

In order to be pharmaceutically acceptable any formulation additive or excipient used in a stable suspension of the invention should be recognized by the FDA as safe for use with humans. Additionally, an excipient should have no effect or minimal effect on the stability of the protein in the ethanol suspension or on the sprayability of the suspensions using and electrostatic spraying means.

Formulations comprising suspensions of protein in ethanol, may be prepared using standard crystallization techniques, or by means of simply over-saturating the formulation with the protein. These formulations are useful for the stabilization of proteins under storage conditions and under conditions used for therapeutic delivery of various proteins. Crystalline suspensions are also useful for delivering high concentrations (i.e., dosage) of a particular protein in the form of a semi-solid slurry.

In general, the particle size of the protein crystals and/or agglomerates used in the suspensions of the invention, will range from about 0.01μ to about 10.0 μ in diameter. If delivery of the protein to the deep lung is the object, the particle size of the protein should preferably range from about 0.01μ to about 5.0 μ in diameter and more preferably from about 0.01μ to about 3.0 μ in diameter. If delivery of the protein to the upper respiratory tract is the object, the particle size of the protein may range from about 5.0 μ to about 10.0 μ in diameter.

Although it is preferred to use anhydrous ethanol in the suspensions of the invention, one may use "substantially" anhydrous ethanol, that is, ethanol containing up to about 5% or less of water and preferably up to about 3% or less of water in the ethanol.

The method of the invention utilizes a dispensing device for comminuting the liquid suspensions claimed herein. Dispensing devices are known which produce a finely divided spray of liquid droplets by electrostatic means (sometimes referred to as `electrohydrodynamic` means). Electrohydrodynamic ("EHD") sprayers are particularly useful in medicine for the administration of medicaments by inhalation. Various EHD devices are known in the art, as for example, those described in US Pat. No. 6,105,877 and US Pat. No. 6,068,199.

The droplet spray in such EHD devices is generated by applying an electric field to a liquid located at a spray head or spray edge. The potential of the electric field is sufficiently high to provide comminution of electrically charged liquid droplets from the spray head. The electrical charge on the droplets prevents them from coagulating via mutual repulsion.

Prior to inhalation of the aerosol to the patient, it is usually necessary to partially or wholly remove the electric charge from the aerosol droplet spray

produced by the electrohydrodynamic comminution device in a controlled manner.

Although other methods may be used, the principal method used to effect comminution discharge utilizes a discharging electrode having a sharp or pointed edge and located downstream from the spray head of the EHD device. The discharging electrode produces a cloud of charged ions from the surrounding air having an opposite electrical charge of equal magnitude to that on the comininuted liquid spray (aerosol). In use, the ion cloud is attracted towards, collides with, and thereby neutralizes the liquid aerosol spray.

Although the protein/ethanol suspensions of the invention are particularly suited for use with EHD devices as would be recognized by one skilled in the art such suspensions are aerosolizable using other aerosol generating devices such as a nebulizer; see WO 99/44664 which describes a pulmonary dosing system and a method for supplying to a patient a predetermined amount of respirable therapeutically active material.

Various embodiments of the present invention are illustrated by the following examples, which should not be intended to limit the scope of the invention.

Example 1

Lyophilized human insulin, supplied from Roche Biochemical Co., was used. To obtain the desired pH, 100 mg of insulin was first dissolved in 2.667 ml of 10 mM PBS buffer, pH 7.4 (Supplied from Sigma, P 3813); 356 ul of this dissolved insulin was aliquoted into15 ml polypropylene conical tubes, which were later used for the 20 mg/ml suspension samples. To make the insulin suspension samples at a concentration of 1.5 mg/ml, 26.67ul of the dissolved insulin was aliquoted into15 ml polypropylene conical tubes. The conical tubes were then sealed with punctured parafilm, and frozen overnight at ~ -15°C to -20°C. The following day the frozen insulin samples were lyophilized to a fine, free-flowing powder using a bench top Labconco Freeze Dry System with Stoppering Tray. This process was started two or three days prior to the start of the experiment. The 100% ethanolic insulin samples were prepared in duplicates. For each sample, there were four (4) time-points prepared. The

lyophilized insulin powder was suspended in 667 ul of 200 proof ethanol, which was supplied from Spectrum Chemicals. The Day "0" samples were immediately diluted with 9.33 ml of 0.1% TFA so the final concentration of the insulin samples would fit within the HPLC standard curve. The diluted samples were then analyzed by reversed phase HPLC method using standard techniques known to the art. The other time-points were sealed with parafilm. Four (4) different conditions were tested: (i) & (ii) samples were maintained at 37° C with and without shaking; (iii) samples were maintained at 40°C with shaking; and (iv) samples were maintained at room temperature with no shaking. A Labline environmental incubator/shaker was used at 37° C and at 40°C while shaking at 220 rpm. Once the next time-point was reached, the samples were again diluted with 9.33 ml of 0.1%TFA and analyzed for insulin activity. In the preparation of all samples containing insulin, care was taken not to sonicate or vigorously shake any preparation of insulin due to the possibility of precipitation. Inversion was used to gently mix.

Table 1 presents stabilization data derived from utilization of this embodiment of the present invention. Percent activity retained was determined by high-performance liquid chromatography (HPLC).

TABLE 1.

Stabilization of Insulin In 100% Ethanol

Time Period	Temp (°C)	Activity Retained (%)	Shaking (rpm)	pH
2.5 months	37	36	NA	3.0
2.5 months	37	92	NA	5.4
2.5 months	37	93	NA	7.4
14 days	37	82	220	7.4
27 days	40	87	220	7.4
2.0 months	40	55	220	7.4
2.0 months	20	90	NA	7.4

Example 2

A. Deoxyribonuclease I (DNase I), Grade II from Bovine Pancreas, supplied from Roche Molecular Biochemicals, was used. Protease Inhibitor, Sigma P 2714, was prepared by dissolving 1 vial with 100ml of MilliQ water. $CaCl_2$, supplied by Sigma, was made into a 7.5mg/ml stock solution, using MilliQ water. Three (3) mg of DNase I was weighed into glass, type 1 reaction vials. Four time-points were prepared for each sample. The samples were prepared in duplicates at either 10mg/ml or 30mg/ml. For the DNase I samples at 10mg/ml insulin 300ul of 200 proof ethanol was added to the reaction vial. The 30mg/ml DNase I samples were suspended with 100ul of 200 proof ethanol, supplied from Spectrum Chemicals. For Day 0 the 10mg/ml samples were diluted with 9.7ml of Buffer A, while the 30mg/ml samples were diluted with 9.9ml of Buffer A. Buffer A consists of the following chemicals at the specified concentrations, 24mM Hepes (Sigma H 3375), 2.4mM $MgCl_2$ (Sigma M 2670), 2.4mM $CaCl_2$ (Sigma C 3881), 0.06% BSA (Sigma A 9647), and 0.03% Tween 20 (Sigma P 7949) at a pH of 7.7. These samples were then analyzed using a colorimetric method utilizing DNA-Methyl Green (Sigma D 2376) as the substrate. All other time-points were sealed tightly and placed in a drawer protected from light and any agitation until the next assay time-point was reached.

Sample Assay Analysis

A standard curve was made with the following concentrations; 1200 ug/ml, 1000 ug/ml, 800 ug/ml, 600 ug/ml, 400 ug/ml, 200 ug/ml, 100 ug/ml, 50 ug/ml, 25 ug/ml and 12.5ug/ml. The standard curve was on each 96-well micro-titer plate. 50ul of each standard and diluted sample was added in triplicate following a previously devised plate map. To the standards and diluted samples 150ul of 0.08 % DMA-Methyl Green was added. Each diluted sample had its own blank, which contained everything the sample did except the DNase I. These were also put on the plate, however 150ul of Buffer A was added to these wells instead of DNA-Methyl Green. After all wells contained the appropriate solutions, it was sealed and placed on a VWR orbital shaker for 1 hour of incubation.

During this hour incubation a stop solution was prepared, 50mM H_2O_2 /50mM EDTA was prepared in water. After the 1-hour incubation period was over 50ul of the stop solution was added to each well. The plates were recovered and again set on the orbital shaker for an addition 2 hours of incubation.

At the end of the total 3 hours of incubation, the plate was read using Molecular Devices, SpectraMAX 380 micro-titer plate reader. The standards were fit to a 4-parameter curve using Molecular Devices, Softmax Pro® version 3.1.2. This software was then used to analyze the data correlating the absorbencies of the unknown samples to the absorbencies of the standards and then finding the concentrations.

The stability data for DNase I is summarized in Table 2. For comparison, data for Dnase I in its standard storage buffer (Hepes) and for a commercial product (Pulmozyme) are included in Table 2. Pulmozyme® (dornase alfa) Inhalation Solution is a sterile, clear, colorless, highly purified solution of recombinant human deoxyribonuclease I (rhDNase), an enzyme which selectively cleaves DNA. Pulmozyme is administered by inhalation of an aerosol mist produced by a compressed air driven nebulizer system. Pulmozyme is marketed by Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990.

Table 2.

Stability Data for DNase I

Biomolecule	Solvent or Solvent System	Time Period	Activity Retained (%)	Temp (°C)	Shaking (rpm)	pH
DNase I	100% Ethanol	2 Months	77%	20	NA	3.0
DNase I	100% Hepes	1 Day	0%	20	NA	6.5
DNase I	Pulmozyme	28 Days	48.6%	20	NA	6.5

The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications

as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.